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Antisense-mediated redirection of mRNA splicing

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Abstract. Antisense technology has been used to study basic biological processes, and to block these processes when they deleteriously lead to human disease. A separate, equally important application of antisense technology is to upregulate the gene expression lost in the diseased state by shifting alternative splicing of pre-messenger RNA. This strategy has commonly relied upon the use of antisense oligonucleotides; however, another approach is to use a plasmid construct to generate antisense RNA inside the cell. Antisense therapeutics based on expression vectors and viral vectors offers a gene therapy approach, whereas those based on oligonucleotides offers a more drug like approach.

Key words. Antisense; splicing; oligonucleotides; snRNAs; gene therapy.

Alternative pre-mRNA splicing splicing

Alternative splicing is emerging as an extremely important process that mediates diversity in the proteomes of higher organisms by generating arrays of protein isoforms. In the most extreme examples, the slowpoke Ca^{++} ion channel [1] and the *Drosophila* gene dscam [2] can be potentially spliced into 500 and 38,000 isoforms, respectively. Since in humans 35–60% of genes are alternatively spliced, this process must account for a large fraction of the estimated 140,000 proteins present in human cells [3, 4].

The mechanism of alternative splicing is not well understood, as evidenced by the fact that attempts to predict legitimate splice sites in silico still generate substantial false positives/negatives [5–7]. While consensus sequences of the elements that control splice site selection, such as the 3′ and 5′ splice sites, the branch point and the polypyrimidine tract are well defined [8], under certain conditions sequences that only very loosely match the consensus sequences can be efficiently used [9]. More recent findings indicate that the function of the above elements may be modulated by exon and intron splicing enhancers and silencers, which contribute to the plasticity of pre-messengers RNA (mRNA) splicing [10]. As a result, splicing of pre-mRNA is not fixed, leading to alternative outcomes controlled by the cell. Importantly, this alternative splicing can also be controlled by external manipulation by antisense RNA and oligonucleotides.

Antisense RNA and oligonucleotides bind to the target pre-mRNA in a sequence-specific fashion, sterically blocking targeted splice sites and redirecting the spliceosome to available and unhindered splice sites. Thus, the antisense molecules act as silencers of the targeted splice sites and thus as enhancers of the alternatively selected sites. This application of antisense technology has clinical relevance, since many diseases are the result of misguided alternative or aberrant splicing [11, 12].

The role of splicing in disease

An annotated database survey showed that up to 15% of point mutations contributing to genetic diseases damage or modify splice sites and other sequence elements involved in splicing, and therefore result in aberrant splicing of pre-mRNA [10, 13]. This percentage could be much higher if it were based on RNA expression and splicing patterns rather than solely on genomic sequence. For example, when analyzed at the RNA level, 50% of

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mutations in the ataxia-telangiectasia and neurofibromatosis type 1 [14] genes resulted in defective splicing. Likewise, of the more than 900 sequence alterations detected in the cystic fibrosis transmembrane regulator (CFTR) gene, about 20% are splicing mutations [3]. Aberrant splicing may be caused not only by a defect in the existing splicing elements but also by mutations that create additional splice sites and/or activate cryptic splice sites. β Thalassemia, a hereditary blood disorder, provides a good example of this mechanism and will therefore be discussed in more detail below [15] and will therefore be discussed in more detail below. In addition, diseases caused by inappropriate splicing may be due not only to mutations in the given pre-mRNA, but also by factors that influence alternative splicing. For example, alternative splice site selection is changed in transformed cells, as demonstrated by the specific alternative splice variants of various pre-mRNAs expressed by many cancers [16].

Modification of splicing by antisense antisense RNA

Close to 200 mutations cause β thalassemia, but those that create additional, aberrant splice sites in introns 1 and 2 of the β-globin gene are among the most frequent worldwide [17]. This laboratory showed that blocking the aberrant splice sites with either antisense RNAs [18–21] or oligonucleotides [22–24] restores correct splicing and correct expression of β-globin and consequently hemoglobin in treated cells.

Figure 1 illustrates that mutations at nucleotides 654, 705 or 745 in intron 2 of the human β-globin gene activate aberrant 3′ and 5′ splice sites within the intron and prevent correct splicing of β-globin pre-mRNA, resulting in inhibition of β-globin synthesis and in consequence β-thalassemia. Treatment of the HeLa or K562 cells that stably expressed thalassemic genes with U7 and U1 small nuclear RNAs (snRNAs), modified to contain sequences antisense to the aberrant splice sites, resulted in reduction of the incorrect splicing of pre-mRNA and a concomitant

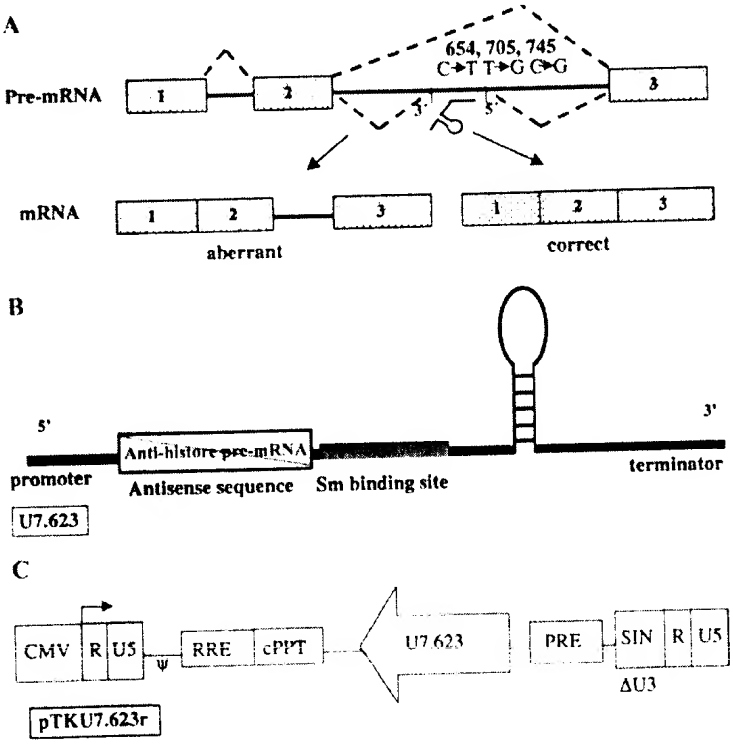

Figure 1. (*A*) Correction of splicing of β-globin pre-mRNA by modified snRNAs. Boxes, exons; lines, introns. The dashed lines represent correct and aberrant splicing pathways. (*B*) Structure of modified U7 snRNA constructs. Wild-type U7 snRNA includes a stem-loop structure, the U7-specific Sm sequence and a sequence antisense to the 3′ end of histone pre-mRNA. The promoter and 3′-terminator regions are indicated. In modified U7 snRNAs, the Sm and antisense sequences were replaced with the spliceosomal Sm sequence SmOPT and with antisense sequences targeted to the β-globin pre-mRNA. The SmOPT site is boxed and the antisense sequences are underlined. (*C*) Lentiviral vector design. The modified U7.623 snRNA was inserted between the central polypurine track of HIV-1 (cPPT) and the downstream long terminal repeat (LTR) of the pTK134 plasmid in reverse (pTKU7.623r) orientation. Transcription of the full-length vector RNA was driven by human cytomegalovirus (CMV) promoter. The vector also contains a packaging signal (ψ), the Rev response element (RRE), a sequence containing the woodchuck hepatitis virus post-transcriptional regulatory element (PRE) and a self-inactivating (SIN) deletion in the U3 region of the downstream LTR. Modified from [21].

increase in correct splicing, which ultimately led to increased levels of the correct β-globin protein [18–20]. This result demonstrates the advantages of targeting splicing: by definition, a decrease in one splice variant leads to an increase of its counterpart and results in a major change in the ratio of the products.

To apply this approach to the treatment of primary erythroid cells from thalassemic patients, with the goal of delivering the antisense RNAs to hematopoietic stem cells, the modified U7 snRNA gene was incorporated into a lentiviral vector. Delivery of this construct into HeLa cells expressing the three thalassemic mutants reduced the incorrect splicing of pre-mRNA and led to increased levels of the correctly spliced β-globin mRNA and protein [25]. Importantly, the therapeutic potential of this system was demonstrated in erythroid progenitor cells from a patient with IVS2-745/IVS2-1 thalassemia. Twelve days after transduction of the patient cells with the U7/antisense lentiviral vector, the levels of correctly spliced β-globin mRNA and hemoglobin A increased approximately 25-fold over background (fig. 2). Although this report did not provide direct evidence that hematopoietic stem cells were affected, similar vectors have been found effective in these cells [26]. Thus it is likely that this approach may have long-term effects, holding great promise for the amelioration of disease.

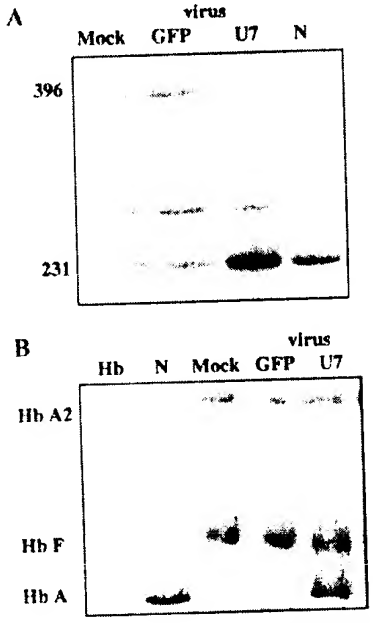

Figure 2. U7.623 lentiviral vector-induced β-globin pre-mRNA repair in erythroid progenitors from IVS2-745/IVS2-1 thalassemic patient. (*A*) Reverse transcription-polymerase chain reaction (RT-PCR). On days 1 and 2 of culture, the cells were transduced with either no virus (lane 1), control, green fluorescent protein (GFP) lentiviral vector (lane 2) or U7.623 lentiviral vector (lane 3). Lane 4, (N) RNA from normal blood. (*B*) Immunodetection of hemolysates separated by electrophoresis on cellulose acetate with anti-human hemoglobin antibody. Lane 1, hemoglobin standards; lane 2, (N) normal blood; lane 3, mock transduced cells; lane 4, cells transduced with GFP lentiviral vector, lane 5, cells transduced with U7.623 lentiviral vector. Modified from [21].

Antisense RNAs encoded in viral vectors have also been used to induce exon skipping in dystrophin pre-mRNA. Mutations that disrupt the dystrophin reading frame lead to premature termination of protein synthesis, and result in Duchenne muscular dystrophy (DMD). In contrast, mutations such as deletions and insertions that maintain the reading frame lead to the synthesis of shorter,defective but semifunctional dystrophin protein, and result in the less severe Becker muscular dystrophy (BMD) [27]. Therefore, if skipping the mutated exon removes the offending mutation and restores the reading frame it should be possible to convert the severe DMD phenotype to the BMD phenotype. This was accomplished by De Angelis et al. who replaced sequences in U1, U7 and U2 snRNAs with sequences antisense to the 5′ and 3′ splice sites of exon 51 in human dystrophin pre-mRNA [28]. These modified snRNA genes were then cloned into the 3′ long terminal repeat (LTR) of the pBabe puro retroviral vector. Viral particles were used to transduce muscle cells from a DMD patient having a deletion encompassing exons 48, 49 and 50. Since this deletion created a premature termination codon in exon 51, skipping this exon partially restored the reading frame of the gene and rescued dystrophin synthesis. The most efficient skipping was obtained when both the 5′ and 3′ splice sites of exon 51 were targeted with antisense molecules. These results indicate that a nonfunctional form of dystrophin mRNA can be converted into a functional form by antisense-induced shifting of splicing patterns.

Modification of gene expression by trans-splicing

Although most pre-mRNAs undergo cis-splicing to form mature mRNA, trans-splicing, whereby sequences from two independently transcribed pre-mRNAs are spliced to form a composite mRNA, also exists. Mansfield et al. [29] modified and enhanced this process, developing spliceosome-mediated RNA trans-splicing (SmaRT) technique. In this technique, fragments of pre-mRNA are designed to base-pair with the intron of a targeted pre-mRNA to enhance trans-splicing between the two molecules, while suppressing cis-splicing of the target. Using SmaRT, this group successfully corrected mutations in cells expressing the mutant form of the CFTR minigene, which contains a mutation (ΔF508) in exon 10. Mature CFTR protein was produced in treated cells, indicating that the newly generated mRNA was translated into correct protein. Recently, Liu et al. demonstrated that SmaRT could correct endogenous ΔF508 mutant CFTR pre-mRNA in cystic fibrosis (CF) airway epithelia and partially restore CFTR-medi-

ated chloride transport. An adenoviral vector was engineered to carry a molecule targeted to CFTR intron 9, allowing for trans-splicing at this locus and replacing the mutated region with the correct one either in vitro in human CF polarized airway epithelia or in vivo in human CF bronchial xenografts [30].

Design of antisense vectors

For the approaches discussed above to be effective, the antisense RNA needs to be delivered in relatively high concentrations. Thus the vectors should include a strong and efficient promoter, which is persistently active either ubiquitously or in a desired tissue. Furthermore, the gene cassette should express RNA molecules that are devoid of any inhibitory secondary structure, colocalize in the same subcellular compartment as the target sequence [31] and be resistant to intracellular nucleases. These criteria are met by snRNAs, making them appealing antisense vectors for modification of splicing.

SnRNAs are localized to the nucleus, are stable and are expressed from their own promoters at relatively high levels. Most importantly despite their structure they interact with their natural pre-mRNA targets by base pairing, i.e. via antisense interactions. Thus, replacement of the antisense sequences with those targeted to a desired pre-mRNA proved effective in the reports discussed above. Analysis of the data from the reviewed reports indicates that U1, U2 and U7 snRNAs are rather similar in their antisense activities. This is surprising since their natural targets and functions are substantially different.

U7 snRNA mediates 3′ end processing of histone pre-mRNA by base pairing the first 18 nucleotides at its 5′ end to the 3′ region of the target [32]. Therefore, U7 can be changed to a mediator of splicing by replacing the anti-histone pre-mRNA sequence with sequences antisense to the target splice sites. U7 snRNA is expressed at low levels (~10^4 molecules/cell), but its nuclear accumulation can be increased by converting the U7 Sm site to the SmOpt sequence shared by more abundant snRNAs [33]. Moreover, this change eliminates U7's function in histone pre-mRNA processing, thus making it available for its new role of modulator of splicing [18]. U1 snRNA is driven by a strong promoter and, because it is transcribed from several genes, its levels reach 10^6 molecules/cell. U1 snRNA is involved in recognition of 5′ splice site sequences, suggesting that it could easily be converted to a modulator of pre-mRNA splicing by changing its binding sequence [32]. However, such a construct will be in direct competition with endogenous U1, which may prevail due to its high concentrations.

Consistent with this hypothesis, in correction of IVS2-705 thalassemic splicing, anti-5′ splice site U1 failed while that targeted to the cryptic 3′ splice was effective in restoring correct splicing of β-globin pre-mRNA [20]. Another possible antisense carrier, U2 snRNA, mediates the recognition of the intronic branch site and the entry of the catalytic U6 snRNA into the spliceosome. Again, this snRNA can be converted to a splicing modifier by replacing its binding sequence with an antisense sequence [28]. These three RNAs are transcribed by RNA polymerase II, while another possible antisense carrier, U6 snRNA, is transcribed by RNA polymerase III. In contrast to the other snRNAs U6 never leaves the nucleus and should be superior in modification of splicing, which proved not to be the case ([20] and M. Vacek, unpublished data).

The best way to deliver the antisense constructs to desired cells is by the use of viral vectors. These vectors are replication-defective viral particles that are able to enter the target cells and transfer their genetic material to the host genome. By replacing the genes required for viral replication with an expression cassette containing the antisense gene, the viruses become safe vectors. Several vectors have been employed to mediate antisense gene delivery, including retrovirus [28], lentivirus [21], adenovirus [30] and adeno-associated virus (AAV) [34]. Retroviral vectors can stably infect dividing cells by integrating into the host DNA; however, they are unable to infect nondividing cells. The human immunodeficiency virus (HIV)-based lentiviruses, in contrast, are able to infect both dividing and nondividing cells. The main disadvantage to these integrating vectors is the possibility of insertional mutagenesis; however, genetic modifications have been made to reduce this concern [35]. Adenoviruses can also infect non-dividing cells, but only confer transient gene expression, as the viral genome remains episomal [36]. Novel forms of these vectors do not induce immune response, which previously led to problems [37]. In contrast, no human pathology has been associated with AAV, which is able to infect certain nondividing cells, such as muscle, brain and liver. However, its use is limited by the small carrying capacity of its genome. AAV provides a very safe vector since it has not been associated with any human pathology [38].

Modification of splicing by antisense oligonucleotides

While antisense RNAs can be expressed for a long time if the constructs are incorporated into the genome [39], antisense oligonucleotides can persist in the cells for at most a few days [40]. Thus, antisense RNA treatments are a form of gene therapy while oligonucleotide treatment represents a pharmacological approach. Oligonucleotides have been used to shift pre-mRNA splicing of CFTR [41], IL-5R [42], c-myc [43], tau [44], SMN -2 [45], and bcl-x [16, 46, 47]. Here we focus on the

shifting of splicing of β-globin and dystrophin pre-mRNA.

β-globin

The mutations at positions 654, 705 and 745 of intron 2 of the β-globin gene (fig. 1) were targeted by 2′-*O*-methyl and morpholino oligonucleotides [11] and morpholino oligonucleotides. Recently, this treatment resulted in the ex vivo correction of β-globin pre-mRNA and up-regulation of hemoglobin in erythropoietic progenitor cells from patients with IVS2-654 and -745 thalassemia [23, 48]. Similar results [48] were also seen in a mouse model of IVS2-654 thalassemia [49]. Although doses of up to 45 μM were required to elicit the effect, sequence specificity and dose and time dependence were maintained. Furthermore, fluorescent-labeled oligomers showed that the compounds were efficiently entering the nucleus of the cells. Similar results were obtained with morpholino oligonucleotides targeted to the β^E aberrant splice site [50].

Dystrophin

As discussed above, antisense-induced skipping of the dystrophin exon containing the premature stop codon can restore the translational reading frame and generate functional protein, converting the severe DMD form to the less severe BMD form of the disease. A mouse model of DMD, carrying a missense mutation in exon 23 of dystrophin gene [51], has allowed the testing of antisense oligonucleotides in vivo. The 2′-*O*-methyl-oligoribonucleotide directed at the 5′ splice site of exon 23 proved effective when locally injected into mouse muscle in the presence of a cationic lipid [52]. Oligonucleotides targeted to an exon splicing enhancer-like sequence induced skipping of dystrophin exon 46 in myotube cells from two DMD patients who carried a deletion of exon 45, which disrupted dystrophin reading frame. Proper translation was restored when the additional exon 46 was also skipped. It was found that a 15% level of exon skipping was sufficient to restore normal amounts of properly localized dystrophin in at least 75% of myotubes [53]. The same group also investigated oligonucleotides targeted to mutations in other forms of the disease [54]. Recently, experiments have been undertaken to optimize the target sequence best suited to restore the reading frame of DMD pre-mRNA [55].

Chemically modified synthetic oligonucleotides

Several requirements must be met for modified oligonucleotides to be effective in shifting splicing: (i) the oligonucleotides must not activate RNase H-mediated destruction of the target pre-mRNA, (ii) they must be able to reach its site of action within the cell [11] and (iii) they must have high enough affinity to the target sequence to effectively compete for binding with splicing factors (reviewed in [11]). Over the past decade, a plethora of such compounds have emerged [56].

2′ Carbohydrate modifications

The addition of residues at the 2′ position of deoxyribose in the oligonucleotide prevents recognition of the oligonucleotide-RNA duplex by RNase H and destruction of the target RNA [57]. 2′-*O*-Me-oligonucleotides were the first to be used to shift splicing [22, 58]. More recently, a 2′-*O*-methoxyethyl (2′-*O*-MOE) phosphorothioate derivative [59, 60] was developed that has higher nuclease stability [61] and improved antisense activity. These compounds also show effects in vivo [62, 63]. Other 2′-modified compounds include the zwitterionic 2′-*O*-aminopropyl oligomer, which harbors a positive charge at the 2′ position in addition to the negative charge on the backbone [64, 65] or locked or bridged oligomers in which a methylene group bridges the 2′-*O* and 4′ positions of the ribose ring [66, 67]. This bridge locks the ribose ring in an N-type conformation, which is more favorable for binding RNA, imparts higher stability, RNase H inactivity and an increase in T_m of approximately +4–5°C/base. Locked oligomers have been used as antisense compounds for shifting splicing [68] or as triplex forming strand invaders [69].

Backbone modifications

Many backbone modifications exist, and most share certain characteristics, such as resistance to cellular and extracellular nucleases, the resistance of RNA:oligomer duplexes to degradation by RNase H and in most cases increased affinity for target sequences. Such oligomers include morpholino [70, 71], peptide nucleic acid (PNA) [72, 73], methylphosphonate (74) and phosphoramidate (NP) oligomers [75, 76]. These oligomers all represent improvements in chemistry and should be useful for shifting splicing.

The EGFP-654 antisense splicing assay

Most antisense assays involve measuring the downregulation of a given mRNA or protein, an approach that is affected by high and possibly variable background signal. Furthermore, nonspecific effects such as toxicity or protein binding can easily be mistaken as antisense effects in these cases. A better assay should be based on a positive signal with low background. The assay method should al-

low antisense testing with high throughput and sensitivity to and yield quantifiable results. Since the nucleus appears to be the major site of action for antisense oligonucleotides [11], the assay should be able to reflect the ability of a given oligonucleotide to accumulate and act in the nucleus. To this end, this lab has developed an assay based on splicing of the IVS2-654 mutant β-globin intron as a positive reporter system for antisense activity [77]. The IVS2-654 intron was inserted into the coding sequence of the enhanced green fluorescence protein (EGFP) gene and prevented EGFP expression due to aberrant splicing that mimics the splicing pathway of IVS2-654 thalassemia. Stable HeLa cell lines that express this construct do not produce EGFP unless splicing is corrected by antisense oligonucleotides. Generation of EGFP after antisense treatment is easily detectable and quantifiable by several methods. This assay provided convincing evidence that the neutral morpholino and PNA oligomers, the latter conjugated to four lysines, outperformed negatively charged 2′-modified phosphorothioate oligomers in free uptake experiments [77].

Recently the above functional assay was recapitulated in vivo in a transgenic mouse model. This model was used to investigate the antisense activity of chemically modified 2′-*O*-MOE phosphorothioates, morpholinos and PNAs containing four lysines at the 3′ end (PNA-4K). It was found that the PNA-4K exhibited the highest antisense activity after systemic delivery, upregulating EGFP in several tissues, including the liver, kidney and heart. PNA oligomers with only one lysine (PNA-1K) were completely inactive, suggesting that the 4-lysine tail is necessary for the antisense activity of PNA oligomers in vivo. The 2′-*O*-MOE oligomers also exhibited antisense activity in the above tissues as well as in the small intestine. The latter effect appears to be related to the intraperitoneal injection of the oligomers. In contrast, little efficacy was seen with comparable concentrations of morpholino oligonucleotides. The sequence-specific ability of PNA-4 and the 2′-*O*-MOE oligomers to upregulate EGFP strongly support that true antisense activity can be obtained by systemically delivered modified oligonucleotides, thus verifying them as potential macromolecular therapeutics. It is also notable that 2′-*O*-MOE were ineffective in cell culture but active in vivo, while the opposite was true for morpholino oligomers. Clearly, further investigations of oligonucleotide uptake mechanisms and the effects of pharmacodynamics on antisense activity are sorely needed.

Analysis of alternative and aberrant splicing with antisense oligonucleotides

Correction of aberrant splicing of IVS2-654 and IVS2-705 pre-mRNAs was observed after treatment not only with oligonucleotides targeted to the aberrant splice sites but also with the one complementary to a region centered around the nucleotide 623 of β-globin intron 2 (i.e. 31 and 82 nucleotides upstream from the aberrant 5′ splice site, respectively) [F. Gemignani, unpublished]. Mutagenesis experiments showed that a four-nucleotide insertion within this region corrected splicing of EGFP-654 [79]. These results suggest that the oligonucleotide or the mutation likely disrupted activity of a splicing enhancer sequence [80–82], thus simultaneously inhibiting aberrant and enhancing correct splicing.

Antisense oligonucleotides were also used to analyze the accessibility of the splice sites in IVS2-654, -705 or -745 pre-mRNAs. Differences in accessibility were demonstrated by treating the cells with a 17-mer antisense oligonucleotide (ON-3′cr) targeted to the common 3′, cryptic splice site activated by the mutations [83]. Although the same site was targeted in all three cell lines, the oligonucleotide exhibited dramatic differences in its EC_{50} (50% efficiency of correction) values for splicing correction in the three contexts. IVS2–654 pre-mRNA splicing was nominally corrected, with the EC_{50} of ON-3′cr at 1500 nM, while for the -705 and -745 pre-mRNAs the EC_{50} values were 30 nM and 2 nM, respectively. Changing the IVS2-654 and -705 splice sites to consensus 5′ splice sites (IVS2-654con, and -705con) led to additional decreases in accessibility of the 3′ splice site to ON-3′cr. IVS2-654con pre-mRNA did not respond to ON-3′cr at any concentration, while IVS2-705con responsiveness was reduced four-fold. The data suggest that the differences in effective concentration must have been due to differences in the ability of the same oligonucleotide to access that cryptic 3′ splice site. These data also support the exon definition model, which postulates interactions between the 3′ and 5′ splice sites to define an exon [84].

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